Reference: *Biol. Bull.* **197:** 188–197. (October 1999)

Behavior of Hemocytes in the Allorejection Reaction in Two Compound Ascidians, *Botryllus scalaris* and *Symplegma reptans*

MAKI SHIRAE[1]*, EUICHI HIROSE[2], AND YASUNORI SAITO[1]

[1] *Shimoda Marine Research Center, University of Tsukuba, Shimoda 5-10-1, Shizuoka 415-0025, Japan; and* [2] *Department of Chemistry, Biology and Marine Science, Faculty of Science, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan*

Abstract. In botryllid ascidians, the type of allorejection reaction differs among species. Comparative studies of these different reactions contribute to our understanding of the allorecognition and nonself-rejection system. We studied the morphology of hemocyte behavior during allorejection reactions in two species, *Botryllus scalaris* and *Symplegma reptans*, which stand at important points in botryllid phylogeny. In *B. scalaris*, phagocytes mediated hemocyte aggregation, resulting in interruption of blood flow just after vascular fusion of incompatible colonies. Although previous studies indicate that morula cells (MCs) play a central role in the rejection reaction, the MCs of *B. scalaris* did not participate in the rejection reaction. Colonies of *S. reptans* showed two types of allorejection reaction that started at different points in the process of vascular fusion between two colonies. In both types of rejection reaction, the MCs played a central role and behaved similarly to those of all botryllids except *B. scalaris* and a botryllid from Israel. These observations suggest that the differences in hemocyte behavior and allorecognition site observed in this study reflect the variation in allorejection reactions among botryllids.

Introduction

Colony specificity is a type of self-nonself recognition and rejection reaction against conspecifics that occurs in many colonial forms of animals; allogeneic colonies fuse to form a single mass or reject each other when the colonies come into contact at their growing edges, whereas syngeneic colonies always fuse with each other. The phenomenon has been studied in various species in different phyla (*e.g.*, Porifera, Cnidaria, Bryozoa, and Urochordata), and the significance of the fusion and the rejection to survival has been discussed. However, it is difficult to reach a reasonable conclusion by comparing the features of colony specificity among a broad range of species because they differ in body plan and physiological condition.

In botryllid ascidians, the morphological process of fusion and rejection reactions in colony specificity has been described in detail for five species: *Botryllus primigenus* (Oka and Watanabe, 1957, 1960; Tanaka, 1973; Tanaka and Watanabe, 1973; Taneda and Watanabe, 1982a, 1982b); *Botryllus schlosseri* (Sabbadin, 1962; Boyd *et al.*, 1990); *Botrylloides simodensis* (Mukai and Watanabe, 1974; Hirose *et al.*, 1988, 1997); *Botrylloides fuscus* (Hirose *et al.*, 1994, 1997); and *Botrylloides violaceus* (Hirose *et al.*, 1988). In these botryllid ascidians, the processes of fusion are essentially the same. When rejection reactions occur, they interrupt the progress of fusion, and the beginning stages of rejection differ among species. That is, the variation in allorejection types is thought to be caused by differences in the allogeneic recognition stage in the colony fusion process (Taneda *et al.*, 1985; Saito *et al.*, 1994).

Hemocytes play important roles in the allorejection reactions of botryllid ascidians (Taneda *et al.*, 1985; Saito *et al.*, 1994). An earlier comparative study on the ultrastructure of hemocytes in some botryllids, including *Botryllus scalaris* and *Symplegma reptans*, showed that hemocytes could be commonly classified into five morphological types: hemoblasts, morula cells (MCs), granular leukocytes, vacuolated cells, and phagocytes (Shirae, unpub. data). Transient cells in differentiation were also observed. We hypothesize that

Received 30 September 1998; accepted 26 June 1999.

Abbreviations: MCs, morula cells.

* To whom correspondence should be addressed. Email: shirae@kurofune.shimoda.tsukuba.ac.jp

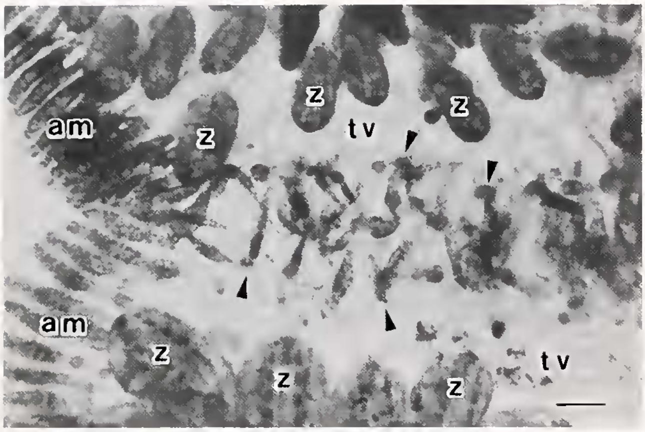


Figure 1. Allorejection reaction in *Botryllus scalaris* under a stereomicroscope. Two incompatible colonies are in contact at their growing edges. Their tunics and ampullae are fused, and hemocyte aggregation in the fused ampullae induces interruption of blood flow (arrowheads). am, ampullae; tv, tunic vessels; z, zooids. Bar = 0.5 mm.

differences in these hemocyte characteristics reflect the variety in allorejection reactions among botryllids.

Botryllus scalaris is thought to be the most primitive botryllid species known to date, based on its manner of sexual reproduction (Mukai, 1977; Mukai *et al.*, 1987; Saito *et al.*, 1981a, 1981b; Saito and Watanabe, 1985). Recently, the molecular phylogeny of botryllids, based on 18S rRNA sequencing, indicated that *B. scalaris* branched off from the lineage earlier than the other botryllids studied thus far (Cohen *et al.*, 1998). In *B. scalaris*, the allorejection reaction begins at the latest stage of the fusion process, that is, soon after fusion of blood vessels and the beginning of blood exchange between two incompatible colonies (Saito and Watanabe, 1982). Therefore, the rejection reaction in *B. scalaris* may be the most primitive type of rejection among botryllid ascidians (Saito *et al.*, 1994). However, there are few descriptions of hemocyte behavior in the rejection reaction to compare with hemocyte behavior of other botryllids.

Symplegma reptans, of the family Styelidae, might be the nearest phylogenetic relative of botryllids for the following reasons. Styelidae is a sister family of Botryllidae (Berrill, 1936), and some researchers (*cf.* Kott, 1969) argue that botryllid ascidians should be classified in a subfamily Botryllinae, belonging to an enlarged family Styelidae. Among compound styelids, *S. reptans* is the species most similar to botryllids morphologically. In botryllids and *S. reptans*, all zooids in a colony are interconnected *via* a common vascular system, and vascular ampullae occur at the growing edge of a colony. Furthermore, the occurrence of colony specificity in *S. reptans* was reported, on the basis of stereomicroscopic observation, by Mukai and Watanabe (1974). They showed that the manner of colony specificity of *S. reptans* resembles that of botryllids.

In this study, we used light and electron microscopy to investigate the processes of allorejection reactions in *B. scalaris* and *S. reptans*, paying special attention to the behavior of hemocytes. In *B. scalaris*, we found that the hemocytes with a central role in the rejection reaction are not morula cells (MCs) but phagocytes. In *S. reptans*, we describe two types of allorejection reaction—the particular combination of allogeneic colonies determines which type occurs. Finally, we discuss the variation in the allorejection reaction in botryllids based on the behavior of hemocytes.

Materials and Methods

Colonies of *Botryllus scalaris* and *Symplegma reptans* were collected in the vicinity of Shimoda (Shizuoka Prefecture), Japan. They were attached to glass slides and reared in culture boxes immersed in Nabeta Bay near the Shimoda

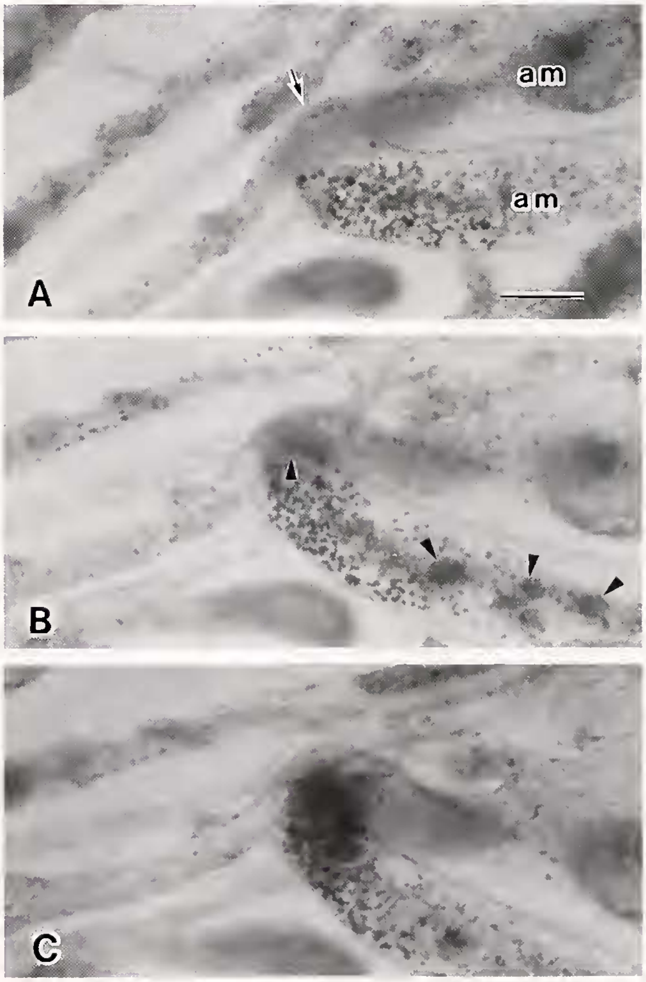


Figure 2. Rejection reaction in *Botryllus scalaris*. (A) Vascular fusion has just occurred and blood of the two incompatible colonies is exchanged. Arrow indicates the fusion point of two ampullae (am) between the incompatible colonies. (B) Hemocytes gradually aggregate in the fused vessels. Arrowheads indicate clusters of aggregating hemocytes. (C) Blood flow is interrupted by the mass of aggregating hemocytes in a few minutes. A, B, and C are the same magnification. Bar = 100 μm.

Marine Research Center, University of Tsukuba. Colonies that grew well were used for the experiments.

Fusion experiments to test fusibility (compatibility) between two colonies were routinely carried out as follows. A small piece was cut from the periphery of each colony with a razor blade. Two allogeneic or syngeneic colony pieces of the same size were brought into contact at their growing edges on a glass slide and were allowed to adhere to the slide in a moisture chamber for 30 min. These colony pieces were subsequently reared in a running seawater aquarium and were observed periodically under a binocular stereomicroscope.

Specimens undergoing fusion or rejection were fixed for 2 h on ice in a 2.5% glutaraldehyde solution containing 0.45 *M* sucrose buffered with 0.1 *M* sodium cacodylate at pH 7.4. The fixed specimens were rinsed in the same buffer and were then postfixed with 1% osmium tetroxide in the same buffer without sucrose for 1.5 h. After dehydration through an ethanol series, the specimens were cleared with *n*-butyl glycidyl ether and embedded in low-viscosity epoxy resins. Thick sections were stained with 1.0% toluidine blue and examined under a light microscope. Thin sections were double-stained with uranyl acetate and lead citrate and then examined with a Hitachi HS-9 transmission electron microscope at 75 kV.

Results

Allorejection reaction in Botryllus scalaris

In *B. scalaris,* as in other botryllids, autogeneic or syngeneic colony pairs fuse and form a single mass. The fusion process was the same as that of other botryllids (Katow and Watanabe, 1980; Saito and Watanabe, 1982). First, the tunics of two colonies fused. Second, the ampullae (termini of blood vessels) penetrated into the opposite colony and the ampullar tips came into contact with the sides of opposite ampullae (tip-to-side contact); at these contact points the colonies fused with each other. Blood started to flow between the two colonies, and finally those colonies formed a single mass. The rejection reaction between allogeneic colonies began after the fusion of opposing ampullae (Fig. 1). Soon after blood exchange through the fused vessels of the two colonies (Fig. 2A), hemocytes aggregated in the vascular lumen of the fused ampullae (Fig. 2B, arrowheads). These aggregates formed a cluster that plugged the opening of the fused ampullae (Fig. 2C) and interrupted the blood flow within a few minutes. Histological observations of the rejection area in the same stage are shown in Figure 3A. As the aggregation gradually proceeded, the volume of hemocyte clusters in the blood vessels increased (Fig. 3, B and C). Subsequently, the vessels collapsed in the rejection area, the surrounding tunic disintegrated, and the two colonies became separate.

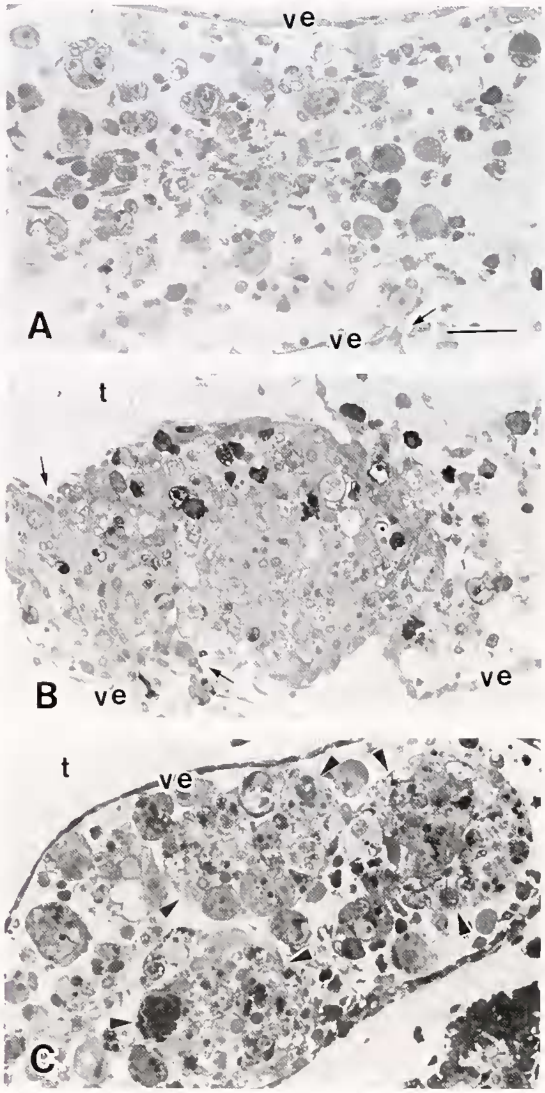


Figure 3. Histological sections in rejection reaction area in *B. scalaris.* (A) Immediately after interruption of blood exchange. Arrow indicates fusion point of vascular epithelia. (B) About 10 h after interruption of blood exchange. Arrow indicates fusion point of vascular epithelia. (C) Two days after interruption of blood exchange. Three clusters encapsulated by phagocytes are shown. t, tunic; ve, vascular epithelia; arrow indicates fusion point of vascular epithelia; arrowheads indicate periphery of hemocyte clusters. A, B, and C are the same magnification. Bar = 50 μm.

The behavior of the hemocytes, as observed by electron microscopy, is shown in Figures 4 and 5. Immediately after the stoppage of blood flow, the cell density of the phagocytes (including hyaline amebocytes, macrophage-like cells, and signet ring cells, as described by Ballarin *et al.,* 1994) increased significantly in the rejection reaction area. The phagocytes had variable shapes, contained many round granules of 0.5 μm in diameter, and often engulfed other hemocytes. Moreover, the phagocytes adhered to other hemocytes and began to aggregate with one another inside the fused ampullae (Fig. 4A). After about half an hour, hemocytes in the aggregates were packed more tightly in the fused ampullae (Fig. 4B), and some of them disintegrated. The aggregates in the ampullae gradually enlarged by

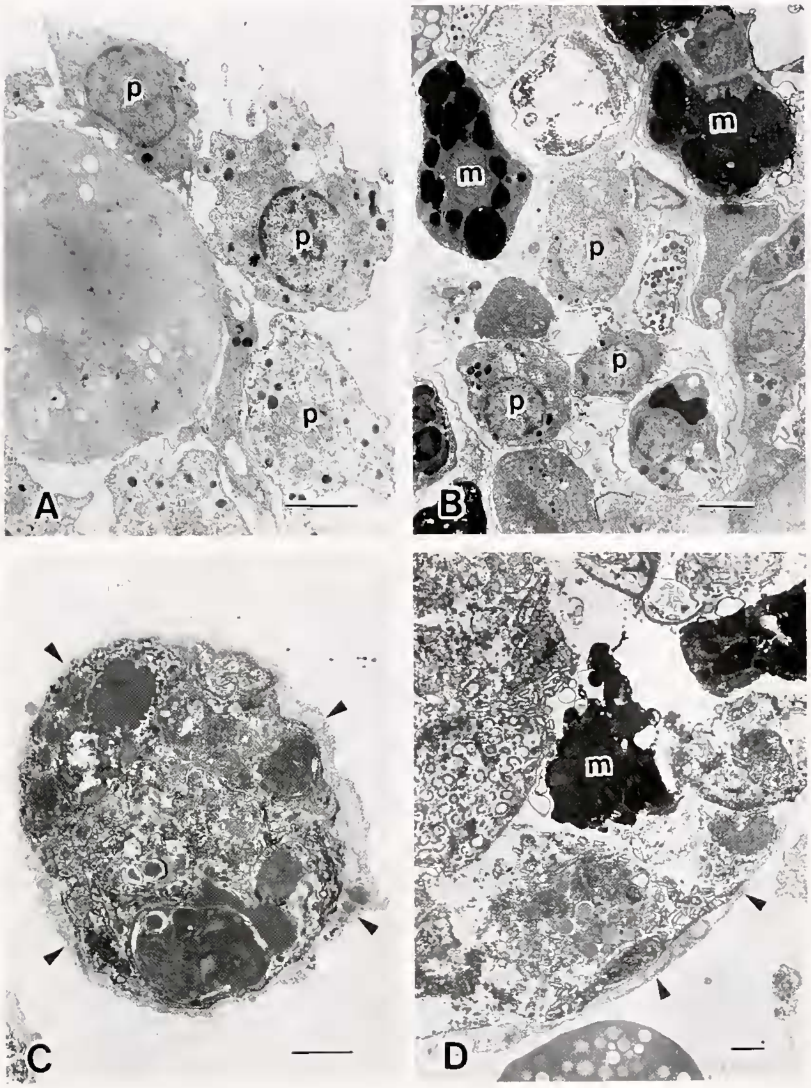


Figure 4. Hemocytes in allorejection reaction in *Botryllus scalaris.* (A) Several phagocytes adhere to a hemocyte. (B) Aggregation. (C) Phagocytosis. Arrowheads indicate outer edges of a phagocyte that surrounded and engulfed a hemocyte cluster. (D) Encapsulation. Arrowheads indicate the periphery of a phagocyte that encapsulated a hemocyte cluster. m, encapsulated and disintegrated morula cell; p, phagocyte. Bars = 2 μm.

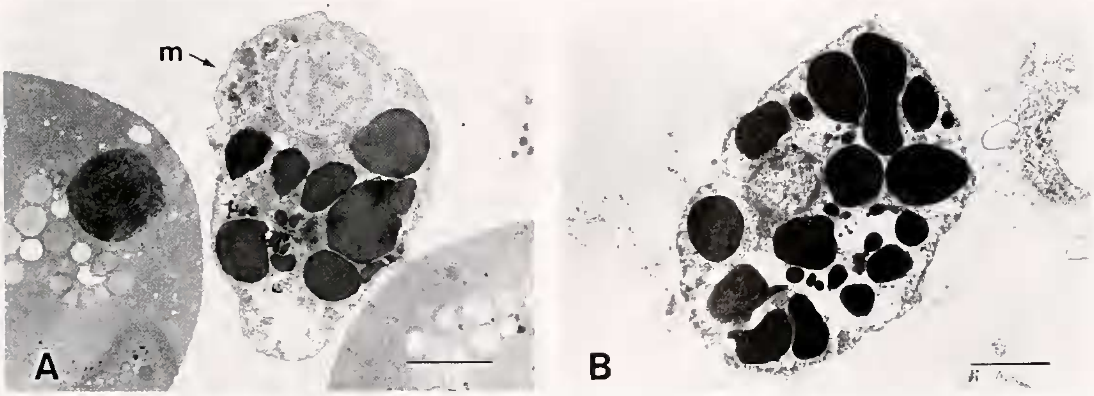


Figure 5. Morula cells in *Botryllus scalaris:* (A) inside the vascular lumen in an allorejection reaction; (B) in an intact colony. m, morula cell. Bars = 2 μm.

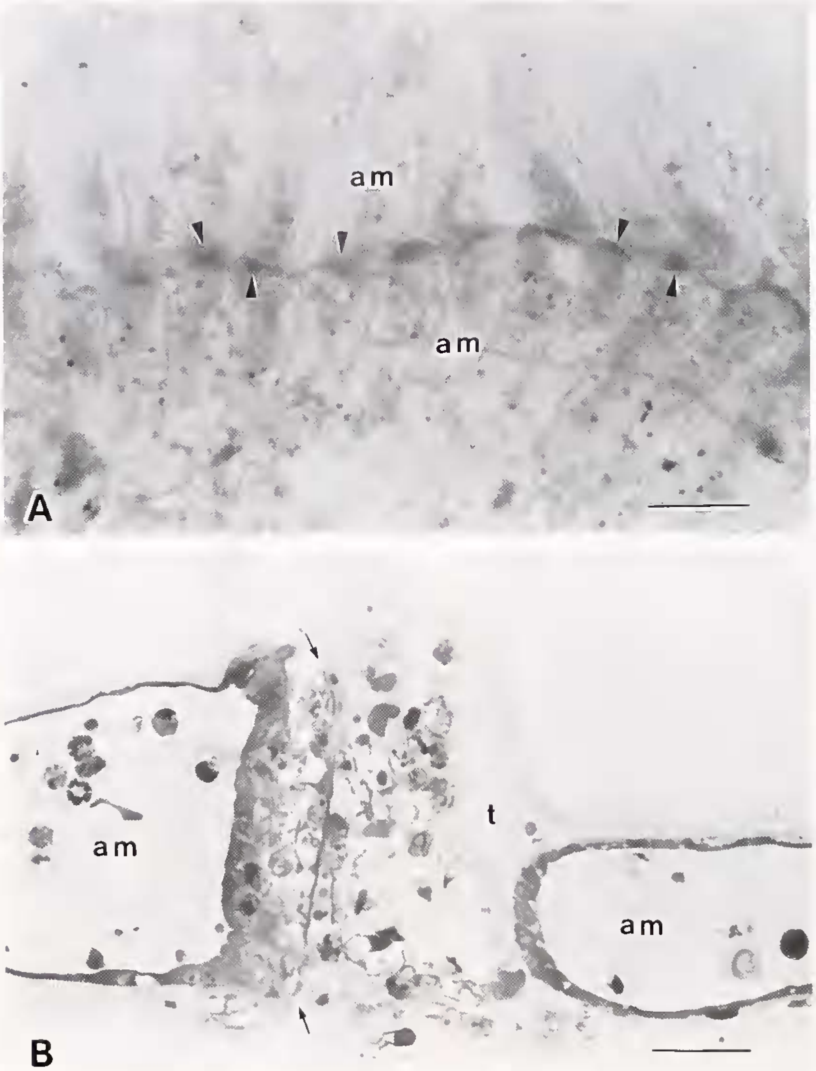


Figure 6. The rejection type of *Symplegma reptans* that is initiated before fusion of ampullae. (A) View of allorejection under stereomicroscope. Ampullae of two incompatible colonies make contact at their growing edges, and infiltrated hemocytes (arrowheads) are observed at the tips of ampullae. (B) Contact area of two incompatible colonies. Arrows indicate the contact point of these two colonies. Many hemocytes infiltrate the tunic and disintegrate, and the surrounding tunic is broken. am, ampulla; t, tunic. Bars: A = 200 μm; B = 50 μm.

recruiting cells from the circulating blood. Furthermore, 3 to 4 h after the stoppage of blood flow, phagocytes engulfed other hemocytes or hemocyte clusters in the rejection areas of blood vessels (Fig. 4C). The hemocyte clusters were encapsulated by phagocytes (Fig. 4D), and the encapsulated clusters sometimes attained a diameter of about 50 μm. All types of hemocytes, including MCs, were engulfed or encapsulated by phagocytes. In the allorejection reaction of other botryllids, MCs always infiltrate the tunic from the blood vessels and disintegrate, but in the allorejection reaction of *B. scalaris,* MCs showed neither infiltration nor disintegration; they also did not show any morphological change inside the blood vessels (Fig. 5).

Allorejection reactions in Symplegma reptans

In botryllid ascidians the ampullae exhibit tip-to-side fusion after extension into the facing colony (Katow and Watanabe, 1980). In *S. reptans,* the fusion process in autogeneic and syngeneic colony pairs has been reported (Mukai and Watanabe, 1974) as tip-to-tip fusion: ampullae of the two colonies did not extend into the facing colony and the ampullae of both colonies came into contact with each other at their tips. But as far as we observed, tip-to-side fusion of ampullae always occurred in the fusion reaction of *S. reptans,* as in botryllid ascidians.

Unlike botryllids, *S. reptans* showed two types of allorejection reaction. The first type was the same as that

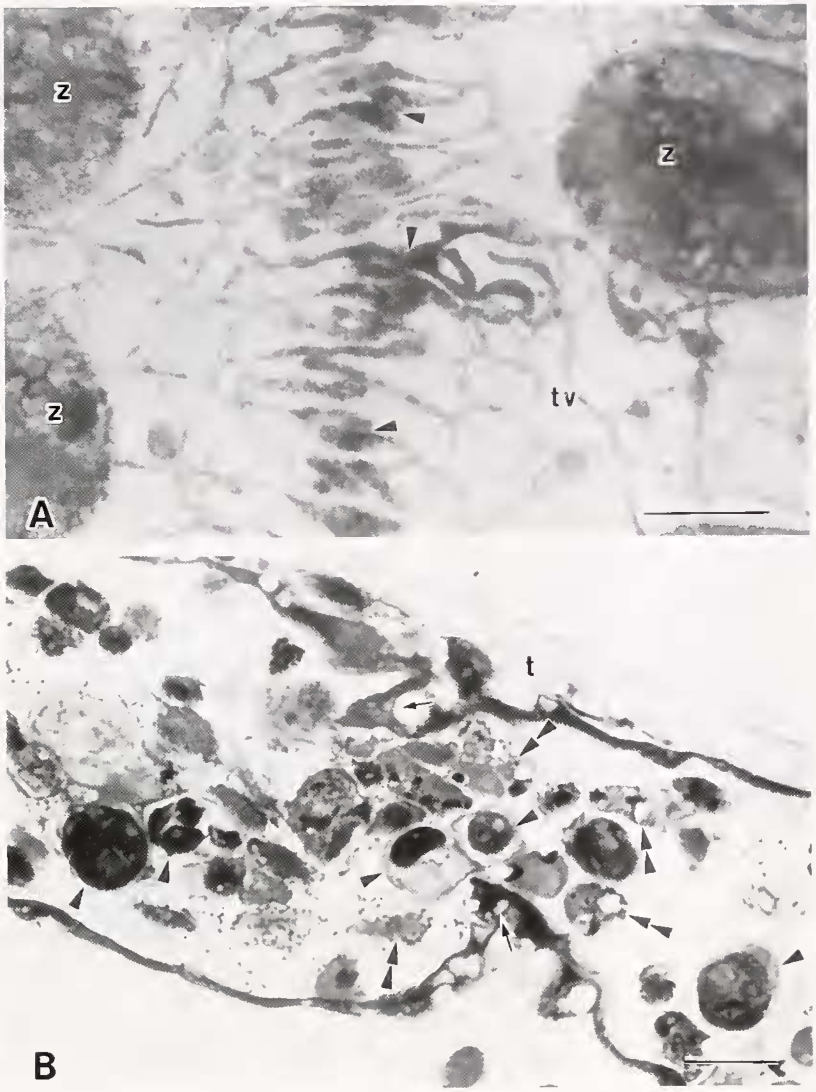


Figure 7. The rejection type of *Symplegma reptans* that is initiated after fusion of ampullae. (A) View of rejection area under stereomicroscope. Arrowheads indicate fused ampullae of two incompatible colonies and aggregation of hemocytes in their ampullae. (B) Inside of fused blood vessels during rejection reaction. Single arrows indicate fused points of vascular epithelia in the two incompatible colonies. Arrowheads indicate phagocytes engulfing other hemocytes. Double arrowheads indicate disintegrating cells. tv, tunic vessels; z, zooid. Bars: A = 500 μm; B = 10 μm.

described by Mukai and Watanabe (1974). In this type of rejection reaction, when two allogeneic colonies came into contact with each other, their tunics fused, but their ampullae neither extended into the opposite colony nor fused with each other. Then, within about 12 h of the contact hemocytes infiltrated the tunic from the ampullar tips at the contact area (Fig. 6A). Then, at the contact area, the tunic around the infiltrating hemocytes disintegrated (Fig. 6B).

In the second type of rejection reaction, which was discovered in this work, fusion of blood vessels and exchange of blood occurred between two incompatible colonies as it does in fusion between compatible colonies. Twelve to fourteen hours after the fusion, hemocytes began to aggregate in blood vessels, and cell aggregation progressed inside the fused ampullae. Thereafter, the vessels were filled with aggregated hemocytes until finally the blood exchange was interrupted completely, within about 24 to 48 h after vascular fusion (Fig. 7A). Histological observations showed phagocytosis and disintegration of cells in this area (Fig. 7B). In addition, hemocytes infiltrated the tunic mainly from the fused ampullae, and these hemocytes and the surrounding tunic disintegrated.

With respect to hemocyte behavior, the two types of rejection reaction were similar. In both types, most of the hemocytes infiltrating the tunic were MCs. During the rejection reaction, MCs disintegrated inside and outside the blood vessels (Figs. 8 and 9), as in some botryllids (such as *Botryllus primigenus* and *Botryllus schlosseri*). Disintegra-

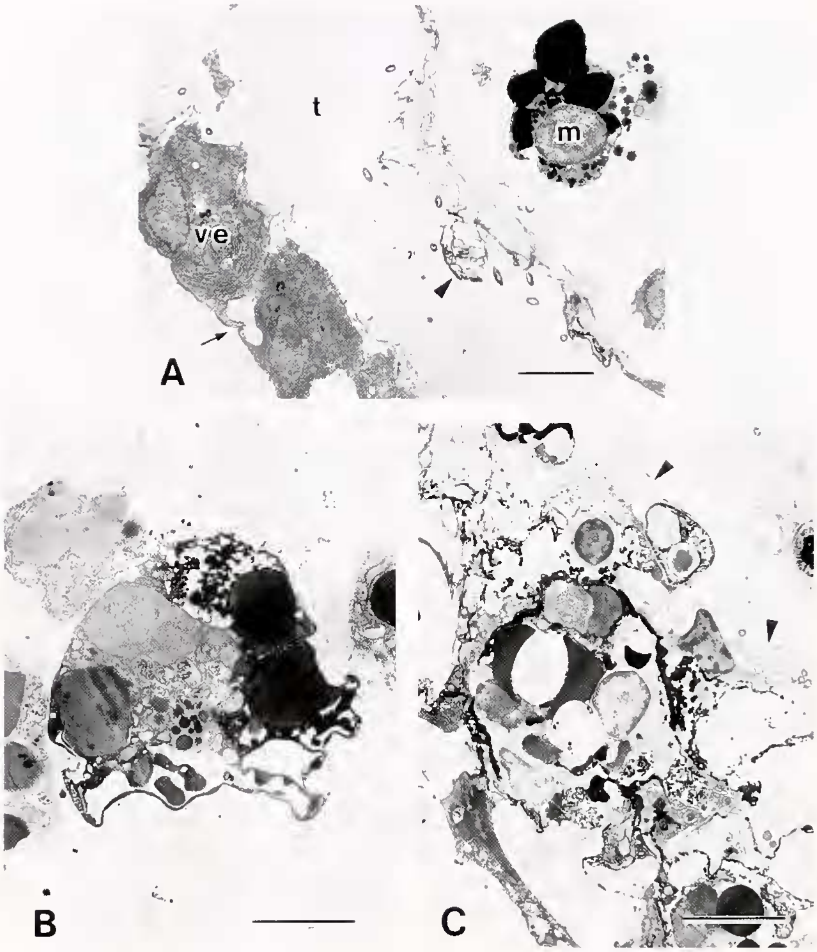


Figure 8. Hemocytes and vascular epithelium in the rejection type initiated before fusion of ampullae in *Symplegma reptans.* (A) Blood vessel and outside of blood vessel. (B) Disintegration of morula cell inside blood vessel. (C) Disintegration of infiltrating morula cell in tunic. Arrowheads indicate electron-dense fiber in the tunic. m, infiltrating morula cell; t, tunic; ve, vascular epidermal cell. Bars = 2 μm.

tion of MCs in the tunic promoted disintegration of the tunic matrix. Phagocytosis occurred in both types of rejection reaction, but encapsulated hemocyte clusters were not found. In the first type of rejection reaction (rejection before vascular fusion), tunic disintegration was limited to the regions surrounding the tips of ampullae in the contact area, and it was more intensive than in the second type (rejection after vascular fusion). However, MC disintegration and phagocytosis inside blood vessels was more common in the second type of rejection reaction than in the first. In the second type of rejection, electron-dense material that might have been discharged from MCs was often found in the vascular lumen (Fig. 9, arrowheads). This material was most abundant in the fused ampullae. The second type of rejection reaction in *S. reptans* progressed more slowly than the rejection reaction of *B. scalaris.* In both types of rejection reaction in *S. reptans,* cellular junctions between the epithelial cells of ampullar tips became loose, and some gaps appeared (arrow in Fig. 8A).

Some *S. reptans* colonies could exhibit both types of rejection reaction, but others showed only the first type (Fig. 10). The fusion experiments with the same pair of colonies were repeated several times and always showed the same results.

Discussion

In five botryllids, *Botryllus primigenus, Botryllus schlosseri, Botrylloides simodensis, Botrylloides fuscus,* and *Botrylloides violaceus,* the rejection reaction between incompatible colonies starts before the fusion of ampullae, although the beginning stages differ among species. These reactions involve activation of a few types of hemocytes, especially MCs, which are ubiquitous hemocytes in ascidians (Wright, 1981). In contrast to these allorejections accompanied by MC activation, the first stage of the rejection reaction in *B. scalaris* was hemocyte aggregation in fused ampullae mediated by phagocytes, especially hyaline amebocytes. The hemocyte clusters clearly caused the interruption of blood exchange between incompatible colonies, as

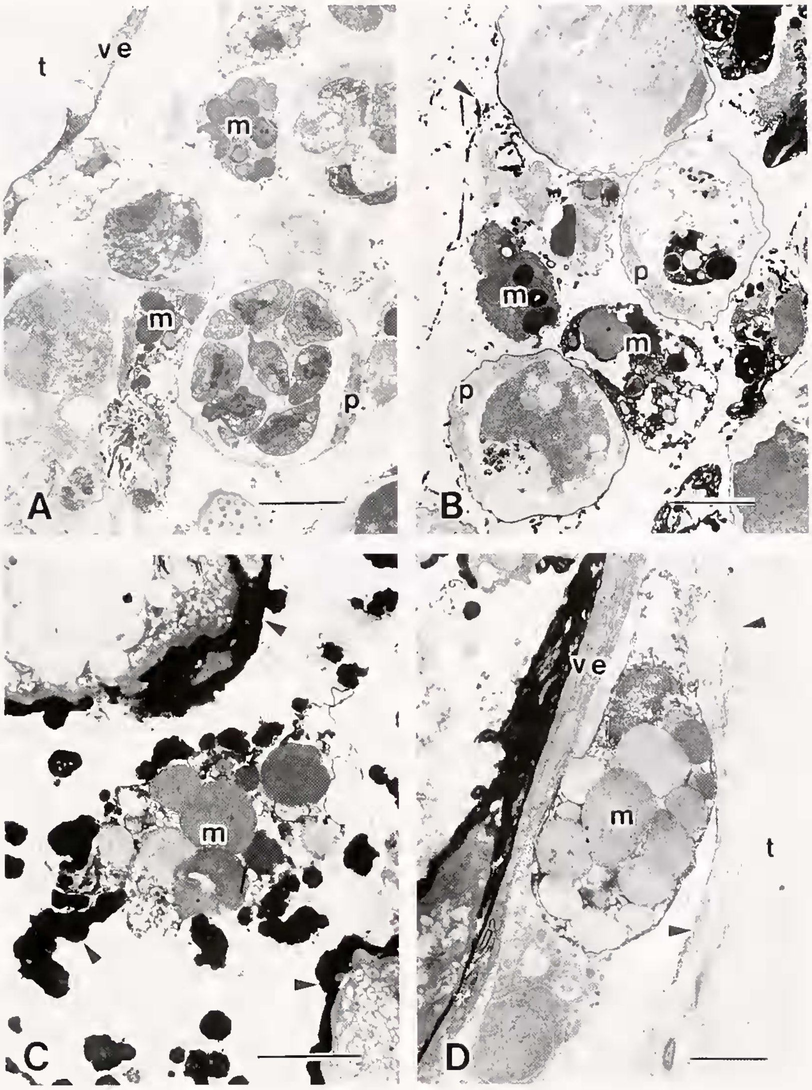
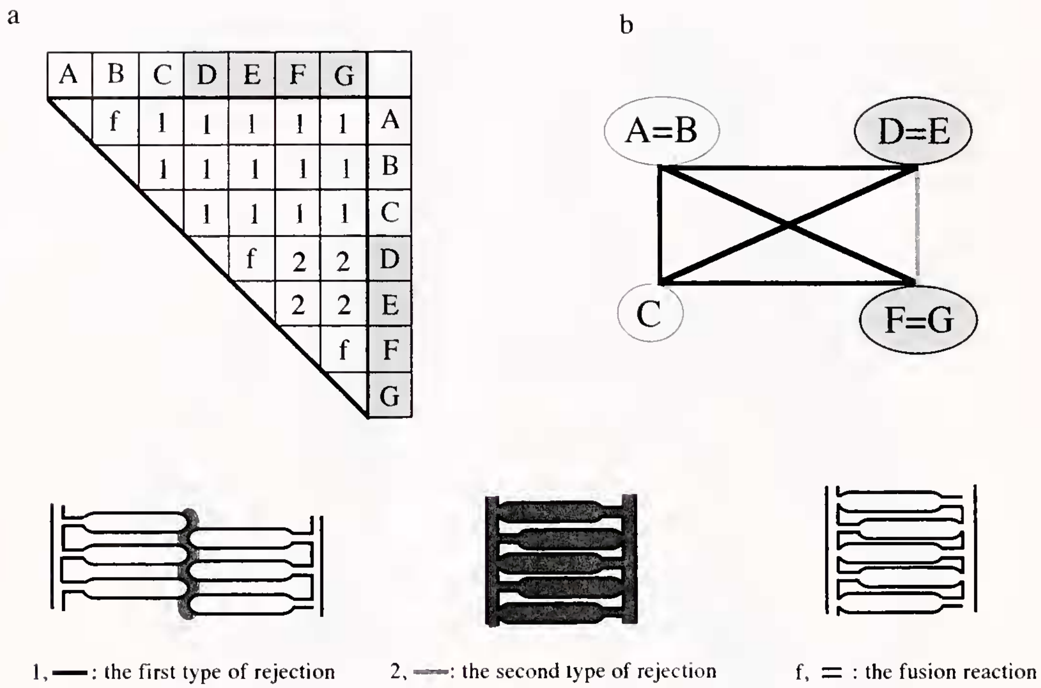


Figure 9. Hemocytes and vascular epithelium in the rejection type initiated after fusion of ampullae in *Symplegma reptans.* (A) Hemocytes in blood vessel where the hemocytes have not aggregated yet. Phagocytosis often occurred. (B) Aggregation of hemocytes mediated by highly electron-dense material. (C) Disintegration of morula cell inside blood vessel. (D) Disintegration of infiltrating morula cell in tunic. Arrowheads in B, C, and D indicate highly electron-dense material inside blood vessel. Arrows in D indicate disintegrated tunic around morula cells. m, infiltrating morula cell; p, phagocyte; t, tunic; ve, vascular epidermal cell. Bars: A and B = 5 μm; C and D = 2 μm.

shown in Figure 2. We labeled this phagocyte-mediated rejection reaction as P-type rejection, to distinguish it from the M-type rejection reaction primarily mediated by MCs in the other botryllids studied thus far. In *S. reptans,* the two types of allorejection reaction shown here could be regarded as M-type rejection, although the second type did not involve remarkable MC infiltration. Morula cell infiltration, which occurs in allorejection in most botryllid species, might be facilitated by the change of permeability in the ampullar epithelium (Taneda and Watanabe, 1982a). This rejection reaction is always accompanied by disintegration of MCs, and their disintegration occurs not only outside but also inside of the ampullae in some botryllids (Rinkevich *et al.,* 1994, 1998; Shirae, unpub. data). Therefore, we regard the standard character of M-type rejection as disintegration of MCs and discharging of their vacuolar contents.

Both MCs and phagocytes are known to have important functions in the defense system of ascidians (Wright, 1981; Raftos, 1990; Cammarata *et al.,* 1997). In the M-type rejection in botryllids and in *S. reptans,* the accumulation of the electron-dense material at the allorejection area is distinctive. Moreover, in the second type of rejection in *S. reptans,* that material seems to mediate hemocyte aggregation, resulting in interruption of blood exchange. Since phenoloxidase activity was demonstrated in MCs (Ballarin *et al.,* 1995), the electron-dense material might be a mela-


a
b
A=B
D=E
C
F=G
1, ▬ : the first type of rejection
2, ▬ : the second type of rejection
f, = : the fusion reaction

A	B	C	D	E	F	G	
	f	1	1	1	1	1	A
		1	1	1	1	1	B
			1	1	1	1	C
				f	2	2	D
					2	2	E
						f	F
							G

Figure 10. A pairwise allorejection assay among seven strains of *Symplegma reptans* (a) and relationships among *S. reptans* colonies concerning allorejection/fusion type (b).

nin-like substance. A recent cytochemical study by Ballarin *et al.* (1998) suggested that phenoloxidase activity of MCs caused cytotoxicity in the allorejection reaction of *Botryllus schlosseri.* However, in the P-type rejection reaction of *B. scalaris,* such a defense system might not act. In *B. scalaris,* phagocytes might involve some functions in mediation of hemocyte aggregation. Consequently, the diversity of hemocyte characteristics among botryllid ascidians may lead to the observed variation in allorejection. Comparative studies of hemocyte histochemistry might further highlight the variation in allorejection systems of botryllids.

A "fusion-rejection" reaction resembling the allorejection reaction in *B. scalaris* was described in an Israeli *Botrylloides* (Rinkevich *et al.,* 1994). In that botryllid, the allorejection reaction started after vascular fusion and involved much phagocytosis, but cell disintegration occurred inside fused ampullae. Because the hemocyte behavior in the Israeli botryllid is unclear, it is unknown whether the rejection reaction is M-type or P-type.

In *S. reptans,* colonies of four strains exhibited the second type of rejection when they contacted and rejected each other (as shown in Fig. 10); colonies of the other three strains always showed the first type of rejection or fusion. Contact between the colonies of the two groups always resulted in the first type of rejection. Thus, the expression of allorejection reaction type might be controlled genetically. In addition, we found that in the second type of rejection, hemocyte aggregation accompanied by accumulation of electron-dense materials was always initiated only at the fused ampullae of the incompatible colonies, although blood was exchanged well through the fused ampullae just after the fusion. The ampullar epithelial cells might lead to the rejection reaction, at least in *S. reptans.* More studies of the two rejection reactions are needed to fully understand the M-type rejection system.

In the present work, we studied the behavior of hemocytes during allorejection reactions, and showed that differences in hemocyte behavior, as well as differences in allorecognition site, reflect the variation in allorejection reactions among botryllids. Differences in hemocyte behavior are probably caused by changes in hemocyte function, in which case the cytochemical and histochemical characteristics of hemocytes may change. Therefore, to understand the allorejection reaction in botryllids, cytochemical and histochemical studies, as well as morphological studies, should be performed using as many botryllid species as possible.

Acknowledgments

This study was supported in part by a grant-in-aid to Y. S. (#08459005) from the Ministry of Education, Science and Culture of Japan. We are grateful to the staff of the Shimoda Marine Research Center (SMRC), University of Tsukuba, for their assistance and hospitality during this work. We also thank Professor K. Tanaka, Dr. T. Otake (Nihon University), and Dr. T. Ishii (Akita University) for their advice. Finally, special thanks to Dr. A. Bellgrove for her kindness in reading the manuscript for grammatical correctness. This report is contribution No. 633 from SMRC.

Literature Cited

Ballarin, L., F. Cima, and A. Sabbadin. 1994. Phagocytosis in the colonial ascidian *Botryllus schlosseri. Dev. Comp. Immunol.* **18:** 467–481.

Ballarin, L., F. Cima, and A. Sabbadin. 1995. Morula cells and histocompatibility in the colonial ascidian *Botryllus schlosseri. Zool. Sci.* **12:** 757–764.

Ballarin, L., F. Cima, and A. Sabbadin. 1998. Phenoloxidase and cytotoxicity in the compound ascidian *Botryllus schlosseri. Dev. Comp. Immunol.* **22:** 479–492.

Berrill, N. J. 1936. Studies in tunicate development. V. The evolution and classification of ascidians. *Philos. Trans. R. Soc. Lond. B.* **22:** 43–70.

Boyd, H. C., I. L. Weissman, and Y. Saito. 1990. Morphologic and genetic verification that Monterey *Botryllus* and Woods Hole *Botryllus* are the same species. *Biol. Bull.* **178:** 239–250.

Cammarata, M., V. Arizza, N. Parrinello, G. Candore, and C. Caruso. 1997. Phenoloxidase-dependent cytotoxic mechanism in ascidian (*Styela plicata*) hemocytes active against erythrocytes and K562 tumor cells. *Eur. J. Cell. Biol.* **74(3):** 302–307.

Cohen, S. C., Y. Saito, and I. L. Weissman. 1998. Evolution of allorecognition in botryllid ascidians inferred from molecular phylogeny. *Evolution* **52(3):** 746–756.

Hirose, E., Y. Saito, and H. Watanabe. 1988. A new type of the manifestation of colony specificity in the compound ascidian, *Botrylloides violaceus* Oka. *Biol. Bull.* **175:** 240–245.

Hirose, E., Y. Saito, and H. Watanabe. 1994. Surgical fusion between incompatible colonies of the compound ascidian, *Botrylloides fuscus. Dev. Comp. Immunol.* **18:** 287–294.

Hirose, E., Y. Saito, and H. Watanabe. 1997. Subcuticular rejection: an advanced mode of the allogeneic rejection in the compound ascidians *Botrylloides simodensis* and *B. fuscus. Biol. Bull.* **192:** 53–61.

Katow, H., and H. Watanabe. 1980. Fine structure of fusion reaction in compound ascidian *Botryllus primigenus* Oka. *Dev. Biol.* **76:** 1–14.

Kott, P. 1969. Antarctic Ascidiacea. *Antarct. Res. Ser.* **13:** 1–239.

Mukai, H. 1977. Comparative studies on the structure of reproductive organs of botryllid ascidians. *J. Morphol.* **152:** 363–380.

Mukai, H., and H. Watanabe. 1974. On the occurrence of colony specificity in some compound ascidians. *Biol. Bull.* **147:** 411–421.

Mukai, H., Y. Saito, and H. Watanabe. 1987. Viviparous development in *Botrylloides* (compound ascidians). *J. Morphol.* **193:** 263–276.

Oka, H., and H. Watanabe. 1957. Colony specificity in compound ascidians as tested by fusion experiments (a preliminary report). *Proc. Jpn. Acad.* **33:** 657–659.

Oka, H., and H. Watanabe. 1960. Problems of colony specificity in compound ascidians. *Bull. Mar. Biol. Stn. Asamushi* **10:** 153–155.

Raftos, D. A. 1990. The morphology of allograft rejection in *Styela plicata* (Urochordata: Ascidiacea). *Cell Tissue Res.* **261:** 389–396.

Rinkevich, B., T. Linker-Levav, and M. Goren. 1994. Allorecognition/xenorecognition responses in *Botrylloides* (Ascidiacea) subpopulations from the Mediterranean coast of Israel. *J. Exp. Zool.* **270:** 302–313.

Rinkevich, B., S. Tartakover, and M. Goren. 1998. Contribution of morula cells to allogeneic responses in the colonial urochordate *Botryllus schlosseri. Mar. Biol.* **131:** 227–236.

Sabbadin, A. 1962. Le basi genetiche della capacita di fusione fra colonies in *Botryllus schlosseri* (Ascidiacea). *Rend. Accad. Naz. Lincei Ser.* **8, 32:** 1031–1035.

Saito, Y., and H. Watanabe. 1982. Colony specificity in colonial ascidian, *Botryllus scalaris. Proc. Jpn. Acad.* **58B:** 105–108.

Saito, Y., and H. Watanabe. 1985. Studies on Japanese compound styelid ascidians. IV. Three new species of the genus *Botrylloides* from the vicinity of Shimoda. *Publ. Seto Mar. Biol. Lab.* **30:** 227–240.

Saito, Y., H. Mukai, and H. Watanabe. 1981a. Studies on Japanese compound styelid ascidians. I. Two new species of *Botryllus* from the vicinity of Shimoda. *Publ. Seto Mar. Biol. Lab.* **26:** 347–355.

Saito, Y., H. Mukai, and H. Watanabe. 1981b. Studies on Japanese compound styelid ascidians. II. A new species of the genus *Botrylloides* and redescription of *B. violaceus* Oka. *Publ. Seto Mar. Biol. Lab.* **26:** 357–368.

Saito, Y., E. Hirose, and H. Watanabe. 1994. Allorecognition in compound ascidians. *Int. J. Dev. Biol.* **38:** 237–247.

Tanaka, K. 1973. Allogeneic inhibition in a compound ascidian, *Botryllus primigenus* Oka. II. Cellular and humoral responses in "nonfusion" reaction. *Cell. Immunol.* **7:** 427–443.

Tanaka, K., and H. Watanabe. 1973. Allogeneic inhibition in a compound ascidian. *Botryllus primigenus* Oka. I. Processes and features of "nonfusion" reaction. *Cell. Immunol.* **7:** 410–426.

Taneda, Y., and H. Watanabe. 1982a. Studies on colony specificity in the compound ascidian, *Botryllus primigenus* Oka. I. Initiation of "nonfusion" reaction with special reference to blood cell infiltration. *Dev. Comp. Immunol.* **6:** 43–52.

Taneda, Y., and H. Watanabe. 1982b. Studies on colony specificity in the compound ascidian, *Botryllus primigenus* Oka. II. *In vivo* bioassay for analyzing the mechanism of "nonfusion" reaction. *Dev. Comp. Immunol.* **6:** 243–252.

Taneda, Y., Y. Saito, and H. Watanabe. 1985. Self or non-self discrimination in ascidians. *Zool. Sci.* **2:** 433–442.

Wright, R. K. 1981. Urochordates. Pp. 565–626 in *Invertebrate Blood Cells.* Vol. 2. N. A. Ratcliffe and A. F. Rowley, eds. Academic Press, London.